PURIFICATION OF THE BRAIN HORMONE OF THE SILKWORM BOMBYX MORI

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The endocrine function of the insect brain was first suggested by Kopeć as early as in 1922 in the gypsy moth, *Lymantria dispar*. Years later, Wigglesworth (1940) clearly demonstrated that the molting of the bug, *Rhodnius prolixus,* was initiated by a hormonal factor originating from the dorsal region of the protocerebrum containing the neurosecretory cells. Since then, numerous studies have clearly defined the function of the insect brain hormone (BH). Thus BH stimulates the prothoracic glands to secrete the prothoracic gland hormone, ecdysone (*e.g.,* Williams, 1947, 1952; Wigglesworth, 1952) which then is thought to act directly on the cells of the various tissues to provoke the growth and metamorphosis of the insect as a whole. BH thus occupies a central position in the endocrine network which controls the post-embryonic development of insects.

The chemical study of BH has been reported from three laboratories with contradictory results. An extract possessing the BH activity was first prepared by Kobayashi and Kirimura (1958) from brains of the silkworm, *Bombyx mori.* Later Kobayashi and his associates (Kobayashi *et al.,* 1962a, 1962b; Kirimura *et al.,* 1962; Saito *et al.,* 1963) obtained the active substance in a crystalline form and identified it as cholesterol. Gersch and his associates (Gersch *et al.,* 1960) obtained the crystalline neurohormones from the entire central nervous tissue of the cockroach, *Periplaneta americana;* the BH activity was detected in one of them, neurohormone D_1 (Gersch, 1961, 1962). In 1960 we obtained a potent watery extract from *Bombyx* brains (Ichikawa and Ishizaki, 1960) and, later, concluded that this active principle was a protein (Ichikawa and Ishizaki, 1963). The present paper deals with the results of the further purification of BH. About 8000-fold purification, on the basis of protein measurement, was achieved and the hormone was proved to consist of chromatographically highly heterogeneous molecules, the molecular weight of the major components varying from 9000 to 31,000.

Materials and Methods

Preparation of assay pupae

Debrained pupae of the Eri-silkworm, *Samia cynthia ricini,* were used for the bioassay of BH. This species is non-diapausing but the arrest of development is brought about when the brain is surgically removed from pupae within 1 day after the pupal molt.

BH was distinguished from the prothoracic gland hormone (ecdysone) by tests on isolated abdomens of *S. cynthia ricini.* The latter were prepared by cutting

pupae into halves between the 3rd and 4th abdominal segments and sealing the posterior half with melted paraffin.

Debrained diapausing pupae of the swallowtail, *Papilio xuthus,* were also used to assay BH. The diapausing pupae were prepared by rearing larvae in a 7-hour photoperiod and the brain extirpation was carried out within several days after pupation. The pupal diapause in this species has been proved to be due to the failure of the brain to secrete BH, as in other lepidopteran species (Ichikawa and Ishizaki, 1958).

Source of BH extract

Bombyx brains were used as extraction material. In the previous communication 5 devitalized *Bombyx* brains were found sufficient to provoke adult development when implanted into a *Samia* assay pupa (Ichikawa and Ishizaki, 1960). To see if a difference exists in the BH activity of the brain at successive developmental stages, 5 completely desiccated brains of *Bombyx* (at the ages of 0, 2, and 4 days after the 4th larval molting, mature larva, prepupa, 0, 3, and 8 days after the pupal molting, and newly emerged adult) were assayed by implanting them into each of the assay pupae. All were active, indicating that no stage-difference existed in the BH activity present in the brain, at least within the range of sensitivity of our assay. Therefore pupae of mixed ages, ranging from shortly after pupation to just prior to adult emergence, were utilized.

Although it was a tedious job to collect the brains, an advantage was the "mechanical" purification, since BH was contained only in the brain in an appreciable titer, as far as examined so far. The "brain" in the present experiments denotes the brain plus a certain amount of neighboring tissues which were not carefully removed in order to reduce the investment of time and labor.

The brains were stored frozen until extracted. They may be stored frozen for at least 1 year with no appreciable loss in BH activity.

Injection

The materials to be tested were injected into assay pupae at the dorso-lateral site of the 4th abdominal segment after anaesthetizing them with ethyl ether. Each pupa received routinely 0.02 ml. of the test solutions. The puncture was sealed with melted paraffin and the injected pupae placed at 25° C.

Penicillin (Kaken Co. Ltd., 200,000 international units/ml. deionized water) and the saturated phenylthiourea (an inhibitor of melanization) were added to the test materials, in a proportion of 5 μl. of each to 0.4 ml. of the test materials. Adding phenylthiourea was essential in the early steps of purification, for the injection otherwise caused occasionally blackening of the blood followed by the death of the assay pupae. Surviving pupae, in this case, often did not develop after injection of BH. This tendency to yield false negative results was markedly enhanced when the extracts were subjected to the first ammonium sulfate precipitation. The reason for this is not clear, but it might be that the extract contains a tyrosinase inhibitor which is removed by the ammonium sulfate fractionation.

TABLE I

Fate of 120 debrained Samia pupae kept at 25° C. after brain removal

Days after operation	0	24	25	27	28	29	30	31	36	57	63	67	70	85	90	100–200	200–300	300–400	400–500
No. emerged as adult moths	—	1	1	—	2	2	2	1	1	1	—	—	1	—	—	2	9	17	15
No. died without apparent adult development	—	—	1	3	—	1	—	—	—	—	1	1	—	1	1	5	14	16	20
No. surviving as pupae	120	119	117	114	112	109	107	106	105	104	103	102	101	100	99	92	69	36	1

Nature of assay pupae, judgment of positive response, and definition of "Samia unit" of BH

The fate of the 120 debrained *Samia pupae* which were monitored for 500 days after brain removal is shown in Table I. Most of them survived for a long time without undergoing adult development, though from time to time some developed spontaneously into brainless adult moths for unknown reasons.

Days required for adult emergence after injection of the materials containing BH varied to some extent, ranging from 17 to 30 days with a mode at 20 days. This period is considerably longer than that between pupation and adult emergence in the normal development, 12–14 days, and was not shortened by high doses of BH. The reason for this delay is unknown but it might be either that a continuous supply of the hormone is necessary for pursuing the normal time course of development or that the metabolic level, once lowered in the debrained resting pupae, needs a supplemental time before recovering the normal level ready for the adult development to start. The moths emerged after injection were of completely adult morphology as far as externally examined, the only anomaly being the failure of the wings to spread out fully.

In the present study, 5 pupae were routinely used for the assay of each experimental lot. The assay result was regarded as positive when more than 3 pupae emerged as adult moths. BH activity was quantified by assaying a series of 2-fold-diluted solutions of the materials. Dilution was made with either deionized water or appropriate buffers. One *Samia* unit of BH was defined as the minimum amount necessary to cause adult development in one assay pupa. An example of the results, to show the mode of response of the pupae to serially diluted BH, is given in Table II. Generally, a boundary concentration (8-fold dilution in this case) where only certain pupae responded could be separated from the clearly positive and negative groups. Thus, in this case, one can know that one *Samia* unit was present in 0.02 ml. of the 8-fold-diluted solution. It is seen that one pupa did not respond to the original solution. Such exceptional individuals were occasionally encountered.

Chemical procedures

All of the chemicals used were of the highest purity commercially available. Aqueous solutions were prepared with deionized water. Sephadex gel (Pharmacia), DEAE-cellulose (Serva, 0.65 mEq/g.), and CM-cellulose (Serva, 0.80 mEq/g.) were washed and packed in columns with standard procedures. Cytochrome *c* and crystalline α-chymotrypsin were the gifts of Dr. T. Kato of the

TABLE II

An example of the assay result to show the mode of response of Samia assay pupae to serially diluted BH

Dilution	No. positive/No. negative	Days required for adult emergence after injection
× 1	4 / 1	19, 19, 20, 20, —
× 2	5 / 0	19, 20, 20, 20, 21
× 4	5 / 0	19, 19, 19, 20, 22
× 8	3 / 2	21, 23, 23, —, —
× 16	0 / 5	—, —, —, —, —
× 32	0 / 5	—, —, —, —, —
× 64	0 / 5	—, —, —, —, —

Department of Botany, Kyoto University, and Eizai Pharmacological Co. Ltd., respectively.

The chemical procedures were carried out either in a cold room (5° C.) or in an ice-water bath. The samples were kept frozen at −18° C. between procedures.

The amount of subtances contained in preparations was expressed in terms of protein amount; protein determinations were made by the use of the Folin phenol reagent with bovine serum albumin as a standard (Lowry *et al.*, 1951). Chromatographic curves were conventionally drawn by the use of optical density at 280 mμ, though even purified preparations did not show a sharp peak at this wavelength.

RESULTS

1. *Purification*

Preparation of crude extract. Preliminary studies on the extractability of BH indicated that it was readily extracted with aqueous solutions, but not with ethyl ether or acetone.

BH was readily extracted with methanol. But after exhaustive extraction overnight with constant stirring with 3 changes of methanol, the residue still possessed an appreciable amount of BH which was readily extracted with saline. This differential extractability will be treated in detail elsewhere. Since all BH was readily extracted with 2% NaCl, this extractant was used in the present study throughout.

One hundred and eighteen thousand brains (fresh weight, 454 g.) were homogenized with 13 times volume (v/w) of acetone and the pellet after centrifugation was dried under reduced pressure. The acetone-dried powder thus prepared (59 *g.*) was extracted 3 times with a total of 2.5 l. of 2% NaCl. Homogenization was performed with a glass homogenizer and centrifugation at 20,000 r.p.m. for 20 minutes. A dark brown extract was obtained. This extract is designated as *Crude Extract.* It contained 992,000 units BH, indicating that one brain contained about 8 units BH.

This extract stimulated adult development also in debrained diapausing pupae of *Papilio xuthus.* The minimum amount necessary to cause development in a *Papilio* pupa was the same as in *Samia,* namely 1 *Samia* unit. Since the average

TABLE III

Purification of BH

Purification step	BH (*Samia* unit)	Protein (mg.)	Specific activity (BH unit/mg. protein)	Purification: —fold	Yield of BH (%)
Crude extract	992,000	16,100	61.5	1.0	100
Heated fraction	980,000	4,900	200	3.2	98.8
Ammonium-sulfate 30–70% fraction	1,120,000	1,400	800	13.6	112.9
First Sephadex G-100 fraction	1,216,000	800	1,520	24.7	122.6
DEAE-cellulose 0.5 *M* fraction	1,280,000	45	28,400	461	129.0
DEAE-cellulose 6–16 fraction	200,000	4.2	47,600	773	20.2
Second Sephadex G-100 fraction-I	34,650	3.25	10,700	174	3.5
Second Sephadex G-100 fraction-II	165,600	0.32	517,500	8,415	16.7
Second Sephadex G-100 fraction-III	149,850	0.36	416,300	6,769	15.1

body weights of *Samia* and *Papilio* pupae were 2 g. and 0.6 g., respectively, *Papilio* was about 3.3 times less sensitive than *Samia*.

This extract failed to cause adult development in the isolated abdomens of *Samia* after injection of as much as up to 50 *Samia* units, excluding the possibility that the active principle was the prothoracic gland hormone.

Heating. The preliminary experiments revealed that the BH activity was resistant to heating at pH around 6. Since the pH of the *Crude Extract* was already 6.5, it was divided into 4 aliquots of equal volume, each of which (620 ml.) was placed in a 2-liter flask and heated in a 90° C. water-bath with vigorous shaking. Protein coagulation began to appear within 5 minutes and the heating was continued for 3 more minutes. Then the extract was rapidly cooled in an ice-water bath and centrifuged. A strongly yellow solution, pH 6.5, was obtained. All of the BH activity was recovered in the supernatant which is designated as *Heated Fraction* (see Table III).

Fractional precipitation with ammonium sulfate. The preliminary experiments of fractional precipitation with ammonium sulfate revealed that the 50–65% saturation fraction contained most of the BH and the 30–50% fraction also contained it in an appreciable amount (Fig. 1).

Based upon the above results, the *Heated Fraction* was subjected to ammonium sulfate fractional precipitation to collect a 30–70% saturation fraction. To the *Heated Fraction* (2.45 l.) 492 g. of solid ammonium sulfate were very slowly added to reach 30% saturation (saturation in this solution at 5° C. was reached by 0.67 g. ammonium sulfate/ml.). Then the solution was allowed to stand for 30 minutes and centrifuged, the precipitate being discarded. Six hundred and fifty-seven g. ammonium sulfate were then added to reach 70% saturation. After centrifugation, the precipitate was taken up with 112 ml. of deionized water. The precipitate readily dissolved to yield a clear, brown solution. This solution was dialyzed for 24 hours

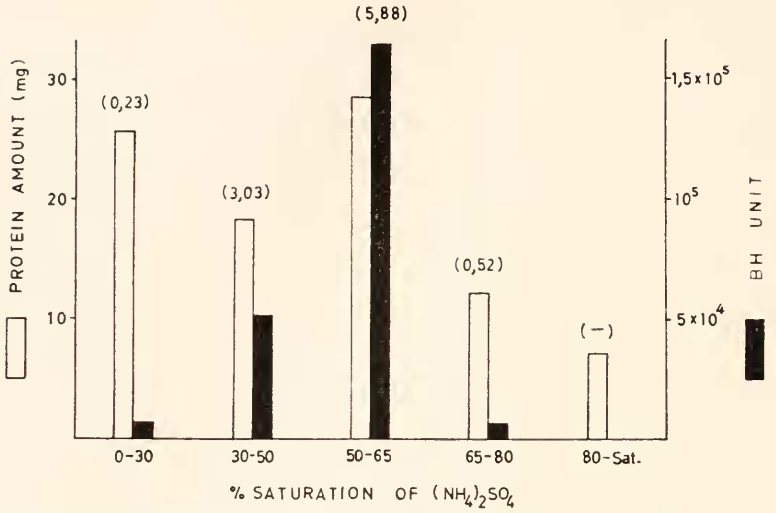

FIGURE 1. Fractional precipitation of BH with ammonium sulfate. The sample was 160 mg. protein containing 180,000 units BH which had been processed through 90° C.-heating of 2% NaCl extract of acetone-dried 23,000 brains. Numerals in parentheses at the top of each histogram indicate the specific activity (*Samia* units/mg. protein).

against 3 changes of 4-liter amounts of 0.05 *M* Tris-HCl, pH 7.8. The addition of ammonium sulfate did not significantly lower the pH of the solution during the above experiment, presumably due to the buffering potency of the extracted substances so that no pH adjustment was necessary.

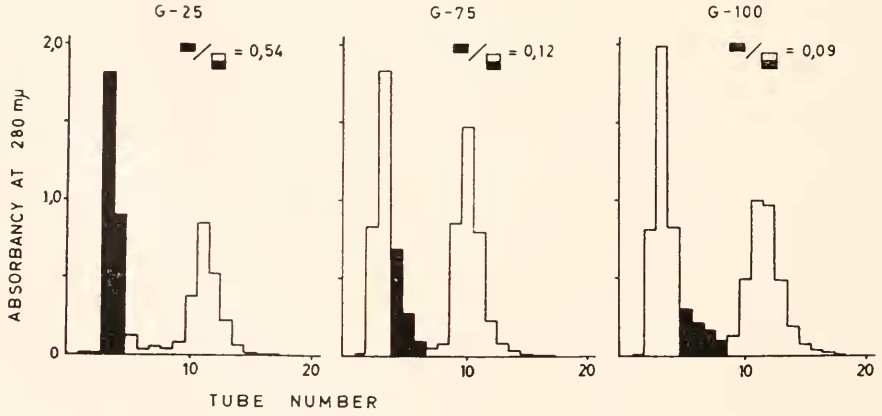

FIGURE 2. Gel-filtration on Sephadex G-25, -75, and -100 columns. Column size, 1.3 × 19.4 cm. Elution buffer, 0.05 *M* Tris-HCl, pH 7.8. Flow rate, 12 ml./hr. Each fraction, 3 ml. The sample for each column was 10 mg. protein containing 40,000 units BH, an aliquot of 30–65% fraction represented in Figure 1. Solid bars represent fractions which contained more than 150 units BH.

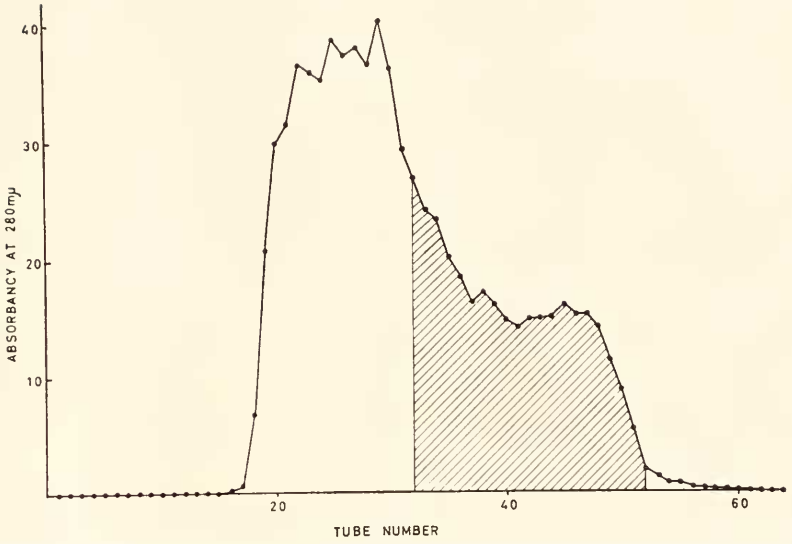

FIGURE 3. First gel-filtration on Sephadex G-100 column. Shaded fractions contained more than 10,000 units BH.

This preparation is designated as *Ammonium Sulfate 30–70% Fraction.* It contained 1,120,000 units BH; the slight rise in total BH units from the preceding step is not significant.

First gel-filtration on Sephadex G-100 column. Preliminary experiments of gel-filtration on Sephadex columns were performed using 3 types of Sephadex, namely G-25, G-75, and G-100. The results are shown in Figure 2. BH was completely excluded from the particles of G-25 whereas partially excluded from G-75 and -100. It is apparent that G-100 is most effective for separation of BH.

The *Ammonium Sulfate 30–70% Fraction* was then subjected to gel-filtration on a 3.7 × 37.0 cm. column of Sephadex G-100. The elution was with 0.05 *M* Tris-HCl, pH 7.8. The flow rate was 60 ml./hr. and 10-ml. fractions were collected. Aliquots from each of the fractions were diluted 20 times with the buffer, and assayed by injecting 0.02 ml. into each of assay pupae. By this assay one can know that fractions which turn out positive must contain more than 10,000 units BH while negative fractions contain less than 10,000. The results are shown in Figure 3. The fractions 32–52 turned out positive. They were pooled and subjected to further purification. This pooled fraction is designated as *First Sephadex G-100 Fraction.*

Stepwise chromatography on DEAE-cellulose. Preliminary experiments indicated that BH bound to DEAE-cellulose at low molarity of Tris-HCl, pH 7.8, while it was eluted with the same buffer of 0.5 *M.* CM-cellulose failed to absorb BH, when applied in 0.01 *M* Tris-HCl, pH 6.5.

The *First Sephadex G-100 Fraction* was subjected to stepwise chromatography on a 2.8 × 36.0 cm. column of DEAE-cellulose. The stepwise elution was performed using 0.05 *M,* 0.14 *M,* 0.3 *M,* and 0.5 *M* of Tris-HCl, pH 7.8, and 0.5 *M* of this buffer containing 0.1 *M* NaCl. The flow rate was 35 ml./hr. and 7-ml. fractions

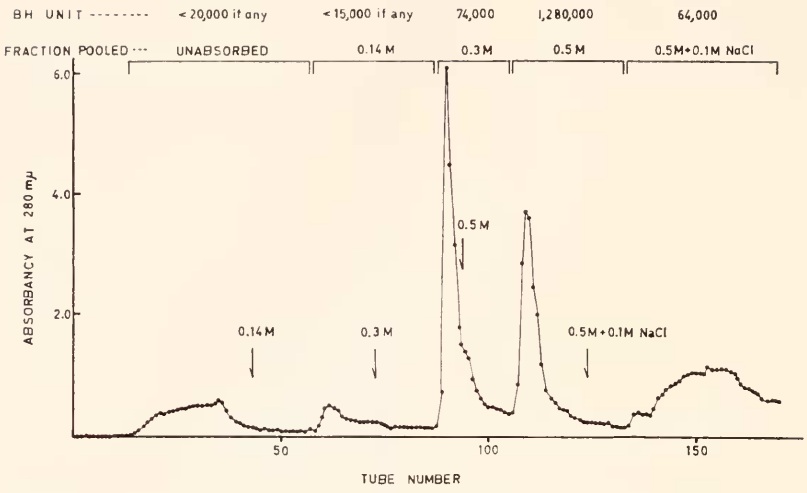

FIGURE 4. Stepwise chromatography on DEAE-cellulose. Tris-HCl buffers (pH 7.8) of the specified molarities were applied at the places designated by arrows. Brackets represent the pooled fractions.

were collected. The effluents at each step were pooled and BH units contained in them were determined. The results are shown in Figure 4. The most active fraction was the 0.5 *M* fraction which contained 1,280,000 units BH. The 0.3 *M* fraction and 0.5 *M* + 0.1 *M* NaCl fraction contained 74,000 and 64,000 units, respectively. The unabsorbed fraction and 0.14 *M* fraction turned out negative when tested by injecting 0.02 ml. into each assay pupa, indicating that they contain less than 20,000 and 15,000 units, respectively. The 0.5 *M* fraction was subjected to subsequent purification and is designated as *DEAE-Cellulose 0.5 M Fraction*.

Gradient chromatography on DEAE-cellulose. The *DEAE-cellulose 0.5 M Fraction* was dialyzed against 0.25 *M* Tris-HCl, pH 7.8, and placed on a 1.9 × 30.0 cm. column of DEAE-cellulose equilibrated with the above buffer. The column was washed with the buffer and gradient chromatography carried out between 300 ml. each of 0.3 *M* and 0.5 *M* Tris-HCl, pH 7.8. The flow rate was 35 ml./hr. and 8-ml. fractions were collected.

Because of the limited number of assay pupae available, the bioassay of 75 fractions was carried out in two steps as follows. First, aliquots from all fractions were diluted 20 times with deionized water, and assayed by injecting 0.02 ml. into each of the assay pupae. By this assay one can distinguish fractions which contain more than 8,000 units BH by their positive responses. After knowing the result of the first assay, the positive fractions only were again assayed to determine the actual amount of BH.

The result is shown in Figure 5. Two discrete groups of BH activity, tubes 6–16 and 23–27, are seen. In addition, the profile of BH activity within each of these groups is irregular, suggesting a high heterogeneity in the molecular form of BH. It should be added further that the washing with 0.25 *M* buffer contained 80,000 units BH. The tubes 6–16 were pooled and only this fraction was subjected

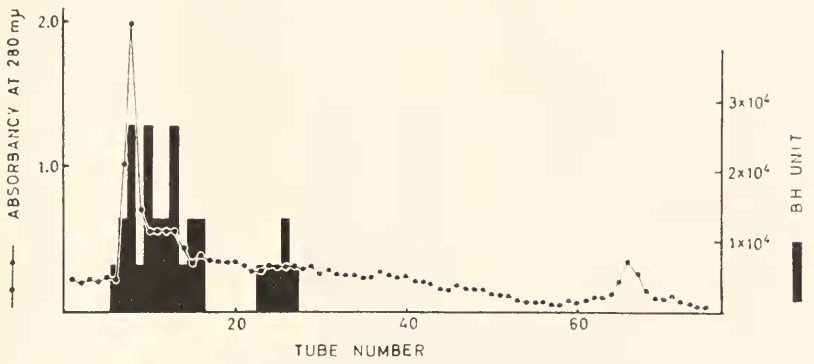

FIGURE 5. Gradient chromatography on DEAE-cellulose.

to further purification. This fraction is designated as *DEAE-Cellulose 6–16 Fraction.*

One may notice that the total activity of BH illustrated in Figure 5 (248,000 units) plus the activity in the washing (80,000 units) is much smaller than that of the starting material in this chromatography (1,280,000 units). However, the first assay employed here implies a possibility that each of the negative fractions might have contained up to 4000 units BH. If all of the 59 negative fractions had contained 4000 units, then $4000 \times 59 = 236{,}000$ units could have been present in these fractions. A sum of all of the above is 564,000. In view of the experimental error necessarily involved in the assay method using 2-fold-dilutions, the difference between 1,280,000 and 564,000 units is in the range of the experimental error. But the above calculation is based upon the assumption that the maximum possible amounts were present in all of the negative fractions. The actual amount in the negative fractions is possibly much smaller: in that case the total amount of BH recovered is too small to ascribe to the experimental error. It is possible that partial inactivation of BH occurred in this purification step.

Second gel-filtration on Sephadex G-100 column. The *DEAE-Cellulose 6–16 Fraction* was precipitated with ammonium sulfate, with pH adjustment at 6.4–6.8 with 0.1 N NaOH. The precipitate was dissolved in 3 ml. of 0.05 *M* Tris-HCl, pH 7.2, and dialyzed against the same buffer. This solution was again subjected to gel-filtration on a 2.8 × 32.0 cm. column of Sephadex G-100. The elution was performed with the above buffer at a flow rate of 30 ml./hr. Fractions were collected each having a volume of 9 ml. Bioassay was again carried out in two steps. In the first step all of the 32 fractions were assayed by injecting 0.02 ml. into each assay pupa. Tubes 4–28 were positive, indicating that they contained more than 450 units BH. The actual amounts of BH were determined only for these positive fractions. The result is shown in Figure 6. The profile of BH activity again suggested the presence of highly heterogeneous components of BH. Tubes 4–16, 17–21, and 22–28 were pooled separately and designated as *Second Sephadex G-100 Fraction-I, -II,* and *-III,* respectively. It is apparent that all of these fractions were still accompanied by considerable amounts of other materials,

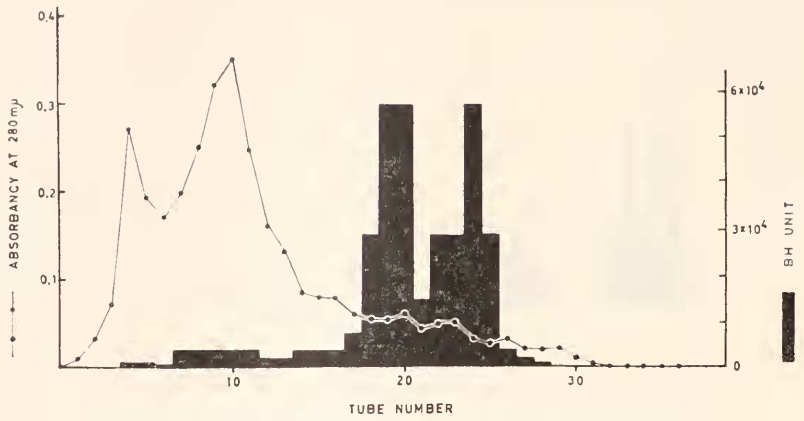

FIGURE 6. Second gel-filtration on Sephadex G-100 column.

in view of lack of correspondence between the profile of BH activity and optical density at 280 mμ.

2. *Estimation of approximate molecular weights of the Second Sephadex Fractions*

The elution of proteins from Sephadex columns is correlated with their Stokes radii and, if the molecules are globular, with their molecular weights (Siegel and Monty, 1966). Based upon this fact the approximate molecular weights of impure preparations of proteins can be estimated by means of Sephadex gel-filtration (Andrews, 1964; Siegel and Monty, 1966).

The molecular weights of the *Second Sephadex G-100 Fraction-I, -II,* and *-III* were estimated by this method. The volume of each of the *Second Sephadex Fractions* was reduced to 2 ml. by means of ammonium sulfate precipitation and then used. The substances of known molecular weights used as standard were blue dextran (Pharmacia, M.W., 2,000,000), γ-globulin (Fraction 2 from bovine plasma, Armour, M.W., 180,000), ovalbumin (Merck, M.W., 45,000), pepsin (Merck, M.W., 35,000), α-chymotrypsin (M.W., 22,500), yeast cytochrome *c* (M.W., 12,950), and bromphenol blue (M.W., 670).

A 3.7 × 35.0 cm. column of Sephadex G-100 was used. The elution was performed with 0.03 *M* Tris-HCl, pH 7.6, at a flow rate of 30 ml./hr. Fractions were collected, each with a volume of 7 ml. Special care was taken to prepare a column which gave good reproducibility on separate runs. To this end, the Sephadex was allowed to swell with deionized water for at least 7 days; it was then packed in the column and the elution buffer was continuously run for 4 days before use. The BH fractions and the standard molecules (10 mg. protein in 1.5 ml. buffer) were separately run and the proteins were read by optical density at 280 mμ, except for cytochrome *c* which was read at 550 mμ. Blue dextran and bromphenol blue were read at 370 mμ. The results were combined and shown in Figure 7. One may notice that the lower molecular-weight limit for complete exclusion of proteins from Sephadex G-100 in the present result is much smaller than that listed in the literature. This may be due either to the difference in lot

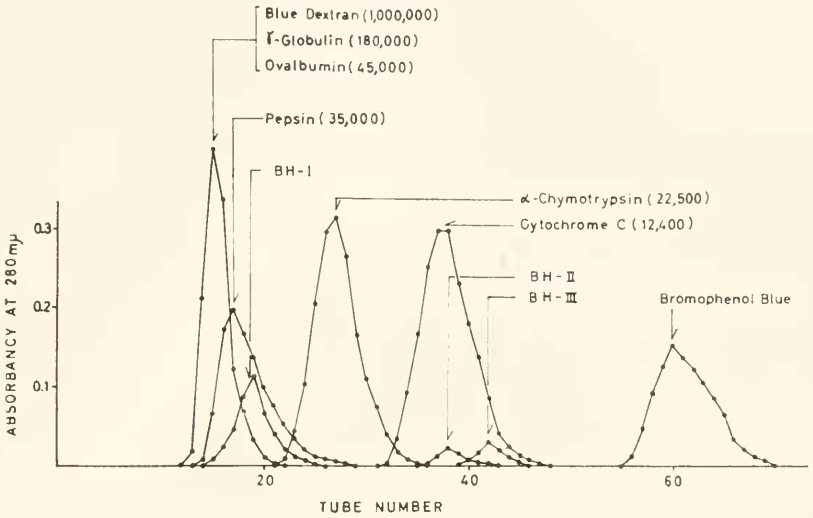

FIGURE 7. Gel-filtration on Sephadex G-100 column of the *Second Sephadex G-100 Fractions* and other substances of known molecular weights. Blue dextran, bromphenol blue, and cytochrome *c* were measured at the wave-lengths specified in text. Blue dextran, γ-globulin, and ovalbumin exhibited similar curves so that only plotting of γ-globulin was presented. BH-I, -II, and -III indicate the *Second Sephadex G-100 Fraction-I, -II,* and *-III,* respectively.

number of Sephadex used or to a slight difference in procedures. In any event, the elution pattern was satisfactorily reproducible between separate runs of identical materials, so that there must have been no defect on the estimation of the molecular weights. The approximate molecular weights of BH were thus estimated as 31,000, 12,000 and 9000 for the *Second Sephadex Fraction-I, -II,* and *-III,* respectively.

DISCUSSION

About 8000-fold purification of BH was accomplished and only 0.002 μg. of the most purified preparation, the *Second Sephadex G-100 Fraction-II,* as determined by protein measurement, was active to cause adult development in a *Samia* assay pupa. The data suggested, however, that the most purified preparations were still accompanied with too many other substances.

The purification procedures employed in this study were all those used routinely for protein purification and BH was successfully purified by these procedures. On the basis of this fact and inactivation of BH by some proteolytic enzymes (Ichikawa and Ishizaki, 1963), we assume that BH is a polypeptide(s) or small protein(s). Recently Kobayashi and Yamazaki (1966) obtained similar results on the proteinaceous nature of BH.

Evidence for the proteinaceous nature of the neurosecretory substances in invertebrates has increasingly been accumulated, conforming to the well established fact that the known neurosecretory substances in vertebrates are polypeptides. Among them are a tanning hormone ("bursicon") in the fly and cockroach brain

(Fraenkel and Hsiao, 1963, 1965; Fraenkel *et al.*, 1966; Mills and Lake, 1966), hyperglycemic and heart-beat-accelerating hormones in the cockroach corpus cardiacum (Davey, 1961; Steele, 1963; Natalizi and Frontali, 1966), heart and hindgut activating hormones in the cockroach corpus cardiacum (Brown, 1965), light-adapting distal retinal pigment hormone and erythrophore-dispersing and -concentrating hormones in the crustacean eye-stalk (Josefsson and Kleinholz, 1964; Kleinholz and Kimball, 1965), and gamete-shedding hormone in the starfish radial nerves (Kanatani and Noumura, 1962, 1964; Chaet, 1964, 1966). The present study on BH adds to the evidence generalizing that the neurosecretory substances are polypeptides or proteins in invertebrates also.

The BH activity manifested highly heterogeneous profiles in DEAE-cellulose chromatography as well as in Sephadex gel-filtration. The heterogeneity in the electric charge as well as in the molecular weight is thus presumed to exist in the molecular form of BH. Three alternative explanations for this heterogeneity are possible: (1) BH itself has multiple molecular forms as in the case of the isozymes or hemoglobin, (2) BH is a single molecule but is associated with other proteins resulting in an apparent heterogeneity, (3) heterogeneity is due to chemical modifications occurring during the purification procedures. But, at present, it cannot be decided which explanation is true.

Kobayashi and Yamazaki (1966) have reported that BH is bound to CM-cellulose but not to DEAE-cellulose. This is precisely the reverse of our results, notwithstanding the use of the same extraction material, *Bombyx* brains and the use of similar buffers. This difference is apparently due to methodology, for the above mentioned investigators did not expose their crude extract to heat or to ammonium sulfate precipitation prior to chromatography.

Kobayashi and his associates (Kobayashi *et al.*, 1962a, 1962b; Kirimura *et al.*, 1962; Saito *et al.*, 1963) have earlier purified an oily substance which can activate the prothoracic glands and identified it as cholesterol. Since then, they have concluded that cholesterol is one of BH, the other being a polypeptide (Kobayashi and Yamazaki, 1966). However, as pointed out by Schneiderman and Gilbert (1964), cholesterol is normally present in the blood at a titer 10,000 times higher than that which is able to cause adult development in the assay pupa when introduced by injection. On the basis of well-documented data concerning the prothoracotrophic activity of the juvenile hormone-mimicking substances, Krishnakumaran and Schneiderman (1965) considered that cholesterol activates the prothoracic glands not because it resembles BH but because it resembles juvenile hormone or ecdysone.

"Neurohormone D_1" or "activation hormone" was obtained in a crystalline form by Gersch and his associates (Gersch *et al.*, 1960) and was proved to activate the ring gland of *Calliphora erythrocephala* (Gersch, 1961, 1962). Reports on the characterization of their substance are awaited. It is also hoped the biological assays with other test animals will be done. The *Calliphora*-test seems inappropriate for the assay of BH, since the dipteran brain-ring gland interaction involves an exceptionally high degree of the neural control (Possompès, 1950, 1958). This may have led to rather ambiguous results in the case of neurohormone D_1.

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Summary

1. The brain hormone extracted from brains of *Bombyx mori* was purified by use of heating, ammonium sulfate precipitation, Sephadex gel-filtration, and DEAE-cellulose chromatography. On the basis of protein measurement, about 8000-fold purification was achieved. The most purified preparation was active by 0.002 μg. protein when injected into brainless pupae of *Samia cynthia ricini*.

2. The brain hormone manifested highly heterogeneous molecular forms which were revealed by Sephadex gel-filtration and by DEAE-cellulose chromatography. The molecular weights of the major components were estimated by Sephadex gel-filtration as ranging from 9000 to 31,000.

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